Physiological and chemical study of the mechanism of chitosan in amelioration of *Lens culinaris* regenerated plantlets under normal and conditions of different salinity levels.

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Abstract

The present study aimed to investigate physiological and biochemical responses of lentil *(Lens culinaris* M.) plantlets to salinity stress concomitant with investigating the role of exogenous chitosan (CHT) application strategy (CHT) in ameliorating the deleterious effect of salt stress .In vitro-produced plantlets of lentil were treated with varying levels of NaCl (50, 100 and 150 mM) added to MS solid media and/or a long with CHT (0.1 mg L^{-1}). lentil shoots and roots of plantlets exposed to NaCl especially (100 and 150 mM levels) exhibited a significant decline in photosynthetic pigments ,carbohydrate fractions. In contrast, lentil shoots and roots sub cultured in salinized MS in vitro and harvested three and ten days later showed increase in proline content, hydrogen peroxide and malondialdehyde. Whereas, addition of CHT (0.1 mg L^{-1}) to the MS media alone or along with different salt levels especially the lowest salt dose significantly mitigated salt stress deleterious effect by enhancing chlorophylls a and b ,soluble sugars, sucrose , starch and proline accumulation manifesting osmotic adjustment.

Keywords: Chitosan, *Lens culinaris* Medik., Plant tissue culture and Salinity

Introduction

Lens culinaris Medik. is the most important food legume worldwide, a leading source of low cost proteins [1]. They satisfy 34.6% of the total protein requirement worldwide [2] and account for over 50% of all legumes consumed globally [3]. Seeds of lentil have other valuable nutritional properties as fiber, minerals, vitamins, and low content of fat and sodium [2]. A diet including lentil provides substantial health benefits, decreasing the risk of heart and renal diseases, protecting against several cancer types [4] and helping in the control of overweight and obesity [5]. Despite its importance, production growth rates are threatened by biotic and abiotic factors; lentil is classified as a salt-sensitive crop so, it is not recommended to intentionally plant lentil in saline soil [6 and 7].

Plant tissue culture is a set of techniques for the aseptic culture of cells, tissues, organs and their components like genes and enzymes under defined physical and chemical conditions *in vitro* and controlled environment. Plant tissue culture technology also explores conditions that promote cell division and genetic re-programming in *in vitro* conditions and it is considered an important tool in both basic and applied studies, as well as in commercial application [8]. Plant tissue culture techniques have become of major industrial importance in the area of plant propagation, disease elimination, plant improvement, and production of secondary metabolites. Chitosan has a broad-spectrum antimicrobial activity against both Gram-positive and gram-negative bacteria [9]. Due to this property, chitosan is a natural antimicrobial agent with potential application in agriculture, food, biomedical and biotechnology fields [10]. Chitosan has been used in the plants to confer resistance against abiotic stresses such as water deficit, salinity, heat stress and heavy metal toxicity [11].

The global population is expected to reach 9.1 billion in 2050 and a 50 percent surge in food demand is expected by that time [12]. It has been projected that more than 50% of yield reduction is the direct result of abiotic stresses [13]. Salinity is a major stress limiting the increase in the demand for food crops in terms of low crop yield and increasing areas not suitable for planting [14]. Another concern is the global climate change that leads to more and more extreme fluctuation of the environmental conditions in agricultural areas [15]. More than 20% of cultivated land worldwide (about 45 hectares) is affected by salt stress and the amount is increasing day by day. Thus, salinity is one of the most brutal environmental stresses that hamper crop productivity worldwide [16] hence, threaten the food security worldwide.

The present study was designed with objectives to evaluate -applying *in vitro* technique- the effect of different concentrations of NaCl, effect of chitosan , and to determine the interactive effects of salinity along with chitosan in terms of changing in certain, physiological and biochemical attributes of *Lens culinaris* Medik. *in vitro* regenerated plantlets. Strategy believed and employed is that, deleterious effects of NaCl can be attenuated by the addition of bio-stimulants (chitosan) looking forward to evoke salt tolerance responses, in addition to elucidate possible mechanism (s) that might be involved in the chitosan promoted responses to salt stress.

Material and Methods

Material

The plant used in this study was purchased from local market in Al Bayda – Libya. The plant was identified and authenticated as *Lens culinaris* Medik. by Botanists in Department of Botany, Faculty science, Omar Al-Mukhtar University, Al Bayda – Libya.

Effect of Chitosan in alleviating salt stress *in vitro* derived seedling:

In this study, tissue culture technique was used: Half basal (2.2) of Murashige and Skoog 1962[MS17] salts nutrient medium with vitamins, glycine and agar supplemented with30 mg L^{-1} sucrose, 0.1 mg L^{-1}myo-inositol is use for *in vitro* seedlings germination. Cultures medium pH were adjusted to 5.8 with 1N KOH or 1N HCl, then with 6g L^{-1} agar prior to autoclaving at 121ºC and 1.2 kg cm^{-2} for 20 minutes. Medium was dispensed as 50 ml per jar (350 ml) and all types of culture media were kept for three days under completely darkness for test of contamination.

Surface sterilization and germination of *Lens culinaris* Medik. seeds:

Seeds of *Lens culinaris* Medik. are washes with running tap water for 30 min. Then they are taken to the laminar air flow surface sterilize by dipping in 70% (v/v) ethanol for 1 min and rinse with sterilize distilled water. Further, they are disinfecteds with 10% (v/v) of commercial clorox (5.25% Cl2) containing two drops of a wetting agent Tween 20 solution for 15 min and rinse three times with sterilize distilled water. In complete aseptic conditions sterilized seeds were picked up with the help of sterilized forceps and inoculated into the culture jars containing the germination medium . Cultures are maintaineds under normal condition (16/8 hours light /dark) and are incubated in a controlled growth chamber at 26±1°C. Old seedlings at the physiological age of 3-4 cm in length with 2-3 of develop leaves (3-weeks old)are subjectseds as a plant materials. Seedling re-culture on full (4.4) MS basal medium augmented with one of the three levels of NaCl (50, 100 and 150 mM) or with 0.1 mg L^{-1} chitosan (CHT, the potent concentration) alone and in combination, different treatments were symbol led from T_1-T_8 as follows:

M_1= MS free CHT and NaCl (control)	M_5 = MS + 0.1 mg L^{-1}CHT
M_2 = MS + 50 mM NaCl	M_6 = MS + 50 mM NaCl + 0.1 mg L^{-1} CHT
M_3= MS + 100 mM NaCl	M_7 = MS + 100 mM NaCl + 0.1 mg L^{-1} CHT
M_4= MS + 150 mM NaCl	M_8 = MS + 150 mM NaCl + 0.1 mg L^{-1} CHT

Sub-cultivation of plants is carried out in saline medium and harvested at different times (3-10 days), and then the plants are transferred from the jars and washed with sterile distilled water. Either of shoots or roots is excised carefully separate and further immerse in liquid nitrogen for 5 min and then stores in refrigerator at -20 ºC until uses.

Physiological and biochemical analyses

Estimation of photosynthetic pigment contents:
Leaf samples (0.2 g) harvested from control and treated plantlets were homogenized in acetone 85% (v/v) following Arnon [18] methoed. Extract was centrifuged at 5,000 rpm for 15 min and absorbance was recorded at 646 and 663 nm for chlorophyll (*a* and *b*) estimation and at 470 nm for carotenoids. Pigment content was calculated according to the following formulae as reported by Lichtenthaler and Wellburn [19]:
Chlorophyll *a* = 12.25 A_{663} – 2.79 A_{646}
Chlorophyll *b*= 21.21 A_{646} – 5.1 A_{663}
Carotenoids = (1000 A_{470} – 1.8 Chl *a* – 85.02 Chl *b*) / 198

Soluble sugars, sucrose and starch extraction and estimation:
Sugars extraction:
To extract soluble sugars, sucrose and starch McCready *et al.,* [20] method with minor modifications was followed. Fresh weighed samples shoot and root (0.3 mg) from all lentil control and treated plantlets, each were macerated in 20 ml of 80 %(v/v) ethanol. The tube was bolted and kept in a boiling water bath for 20 min, and allowed to cool to room temperature. After applying the tube for 10 min in centrifuge apparatus at 10 000 rpm the supernatant was decanted. Again 20 ml of previous ethanol was added to the remaining residue inside the tubes, stirred, boiled, cooled and centrifuged as previously followed and the supernatant was collected and added to the first extract.

Soluble sugars, sucrose and starch estimation:
The anthrone method is a colorimetric method for determining the concentration of total sugars. Sugars react with the anthrone reagent under acidic conditions to yield a blue-green color which had a linear relationship with the sample amount of sugar. This method determines both reducing and non-reducing sugars due to the presence of the strongly oxidizing sulfuric acid [21].

Total soluble sugar estimation:
Total soluble sugar and sucrose were determined following Riazi *et al.,* [21].method, in which of the alcoholic extract (0.5 ml) was gently added in a test tube to 3.0 ml freshly prepared anthrone (150 mg of anthrone and 100 ml of 72% of H_2SO_4) and placed in a boiling water bath until stable color development. Color was read at 625 nm and standard sample of glucose (0.1 mg / ml) were treated alone with treatments for calculations.

Sucrose estimation:
Sucrose content was determined after hydrolyzing 1.0 ml from the alcoholic extract by adding 1.0 ml of 5.4 N KOH and heated for 10 minutes at 100 ºC in water bath [22]. After cooling, 3.0 ml of freshly prepared anthrone reagent was then added and boiled till color development. Color was read at 620 nm sucrose (0.2 mg/ml) was used as standard.

Starch estimation:
Sample (0.1 g) from dried sugar free pellet was resuspended in 2.5 ml of distilled water and subsequently 3.5 ml of 52% (v/v) perchloric acid (PCA) was added to the residue after stirring the mixture, the content was centrifuged for 15 min at 4,000 rpm. The supernatant was decanted, collected and the whole procedure was repeated twice. Supernatant of each step was then hydrolyzed poured and the total volume was made up to 15 ml with distilled water. After filtration, 1.0 ml of the aliquot of this filtrate was analyzed for starch content following the same procedure as that of total soluble sugars. Quantity of starch was calculated in terms of glucose equivalent. The quantity of starch was expressed mg glucose/g DW.

Estimation of total soluble proteins:
Soluble protein was determined by using Folin - Ciocalteau reagent according to Lawry *et al.,* [23] assay. Sample from shoot and root tissue (0.1 g) was homogenized in 10% Trichloroaceticacid (TCA) to precipitate protein. Solutions were centrifuged at 5,000 rpm for 15 min, supernatant was discarded and precipitated in 5.0 ml of 0.1N NaOH.

Reagents required were prepared:
A) 2.0% Na_2CO_3 in 0.1 N NaOH.
B) 0.3% $CuSO_4$ $5H_2O$ in 1% sodium potassium tartrate.
C) Folin's Phenol reagent (Folin - Ciocalteu's) 1N.
D) Standard protein solution: Bovine serum albumin (BSA) 0.1 mg / ml in NaOH.
E) Reagents **A** and **B** in 50:1 were mixed shortly before assay.
Protein content was assayed in a reaction mixture contains 1.0 ml of NaOH and 1.4 ml of solution mixture **E.** Then, the mixture was allowed to incubate at room temperature for 10 min, prior to the addition of 0.2 ml of solution **C.** Samples were mixed immediately with each addition. Color was allowed to develop for 30 min at room temperature and the absorbance measured at 750 nm, protein was expressed as mg protein / g FW.

Estimation of proline content:
Free proline content was determined by means of a rapid colorimetric method using ninhydrin reagent according to Bates *et al.,* [24]. Shoot and root samples (0.1 g)

harvested from control and treated plantlet were homogenized in 5.0 ml of 3% (w/v) sulfosalicylic acid and the homogenate was centrifuged for 15 min at 4,000 rpm. To estimate proline content the supernatant (1.0 ml) was treated with 1.5 ml of acidic ninhydrin reagent (1.25 g of ninhydrin was dissolved in a solvent prepared by mixing 30 ml of glacial acetic acid with 8.0 ml of ortho-phosphoric acid and 12 ml of distilled water), boiled at 90°C for one hour. The reaction was terminated in an ice bath and then 5.0 ml of toluene was added to the mixture in a separating funnel and vortexes well. Then the absorbance of the upper phase was determined at 520 nm and the commercial proline (50 µg / 0.5 ml) was used as standard. The proline content was calculated on fresh weight basis according to the following formulae:

µmol proline g^{-1}FW = (µg proline mL^{-1} * mL of toluene / 115.5) / (g of sample)

Oxidative burst levels:

Lentil tissue samples shoot and root (0.1 g) harvested at selected time intervals from all cultures were macerated in 2.0 ml of 0.1 % (w/v) trichloroacetic acid (TCA) as Heath and Packer [25] method. The macerated samples after centrifugation for 15 min at 4 000 rpm were decanted and used for hydrogen peroxide content and lipid peroxidation determination as two selected parameters to assess the tissues oxidative damage.

Determination of hydrogen peroxide (H_2O_2) content:

A hydrogen peroxide level was estimated according to *Sergiev et al.,* [26]. method. Pipetted 0.5 ml was taken from each sample added to 0.5 ml of 10 mM sodium phosphate buffer pH 7.0 and 1.0 ml of 1.0 M potassium iodide (KI). Absorbance was recorded at 360 nm. Hydrogen peroxide (50 mM ml^{-1}) was used as standard sample and the hydrogen peroxide content was expressed as mmol g^{-1}FW.

Determination of lipid peroxidation:

Malondialdehyde (MDA) considered as the major compound among lipid peroxidation secondary products was estimated by thiobarbituric acid (TBA) assay method following Heath and Packer [25]. method. To 0.5 ml aliquot of supernatant 2.0 ml of 0.5 % TBA (v/v) in 20 % TCA (v/v) was added. The mixture was placed in a water bath and heated for 30 min at 95 °C before transferring quickly to an ice water bath. All tubes were centrifuged at 10 000 rpm for 10 min. and the absorbance of the supernatant was measured at 532 nm. The value for non-specific absorption at 600 nm was subtracted. MDA concentrations were calculated by means of an extinction coefficient of 155 $mM^{-1}cm^{-1}$and the following formula [27].

MDA (µmol^{-1}g FW) = [(A532 −A600)/155] ×10^3×dilution factor

Statistical analysis:

The test of least significant using difference (L.S.D) at the level of 0.05% significance was used to examine differences among treatment means and interactions. Data were statistically analyzed using MSTAT-C software package according to the method described by Freed *et al.,* [28].

Results and Discussion

Changes in photosynthetic pigments:

Photosynthetic pigments content (Chlorophyll *a, b* and carotenoids mg g^{-1} FW) in leaves of *Lens culinaris* Medik. plantlets were significantly decreased with increasing the levels of salinity stress compared to control (Table 1). Plantlets treated with chitosan alone (M_5) caused highly significant increase in photosynthetic pigments in the two

ages referred to control non treated and non-stressed (M_1). The recorded data indicated that chitosan application along with different salt levels successfully alleviates the deleterious effect of salinity stress by improving pigments content in leaves of regenerated plantlets.

Supplementing MS medium with 50 mM NaCl + 0.1 mg L^{-1} Chitosan (M_6) induced the highest significant increase in chlorophyll *a* contents (0.66 and 0.82 mg g^{-1} FW) and in chlorophyll *b* content (0.47 and 0.66 mg g^{-1} FW) during the experiment time 3 and 10 days later treatments (Table 1). The present results showed that the ameliorative effect of chitosan on chlorophyll *a* and chlorophyll *b* contents against salt stress gradually decreased with the gradual increasing salt concentration in the culture media.

The recorded data in Table 1 showed that the application of chitosan enhanced the biosynthesis of carotenoid in the treated plants and this increment was greater at plants treated with chitosan alone (1.15 mg g^{-1} FW) or in combination with the lowest salt level 50 mM (1.22 mg g^{-1} FW) after 10 days of application and compared with non stressed plantlets. On the other hand the enhancement effect of chitosan significantly reduced with increasing NaCl concentration in the cultivated media during the two stages (3 and 10 days).

Table 1: Effect of exogenous amendment of chitosan to MS culture media on photosynthetic pigments chlorophyll *a,* chlorophyll *b* and carotenoids of leaves of *Lens culinaris* Medik. regenerated plantlets under normal and conditions of different salinity levels at 3 and 10 day harvesting time after application.

Parameters / Treatments	Photosynthetic pigments (mg g^{-1} FW)					
	Chlorophyll *a*		Chlorophyll *b*		Carotenoids	
	Harvesting time (day)					
	3	10	3	10	3	10
M_1	0.56^{b}	0.77^{b}	0.38^{b}	0.42^{c}	0.78^{b}	0.80^{c}
M_2	0.49^{c}	0.51^{d}	0.44^{b}	0.38^{c}	0.43^{d}	0.71^{d}
M_3	0.28^{f}	0.33^{f}	0.25^{d}	0.26^{d}	0.33^{e}	0.55^{e}
M_4	0.23^{e}	0.29^{f}	$0.2\,0^{d}$	0.21^{d}	0.28^{e}	0.42^{f}
M_5	0.58^{b}	0.79^{a}	0.43^{a}	0.57^{b}	0.88^{a}	1.15^{b}
M_6	0.66^{a}	0.82^{a}	0.47^{a}	0.66^{a}	1.12^{a}	1.22^{a}
M_7	0.35^{d}	0.69^{c}	028^{c}	0.41^{c}	0.61^{c}	0.75^{cd}
M_8	0.32^{de}	0.44^{e}	0.28^{d}	0.37^{c}	0.53^{cd}	0.78^{d}
LSD at 5%	0.045	0.53	0.031	0.053	0.064	0.074

Means having the same letters in a column were not significantly different at $p<0.05$

Where:

M_1= MS free CHT and NaCl (control)
M_2 = MS + 50 mM NaCl
M_3= MS + 100 mM NaCl
M_4= MS + 150 mM NaCl
M_5 = MS + 0.1 mg L^{-1}CHT
M_6 = MS + 50 mM NaCl + 0.1 mg L^{-1} CHT
M_7 = MS + 100 mM NaCl + 0.1 mg L^{-1} CHT
M_8 = MS + 150 mM NaCl + 0.1 mg L^{-1} CHT

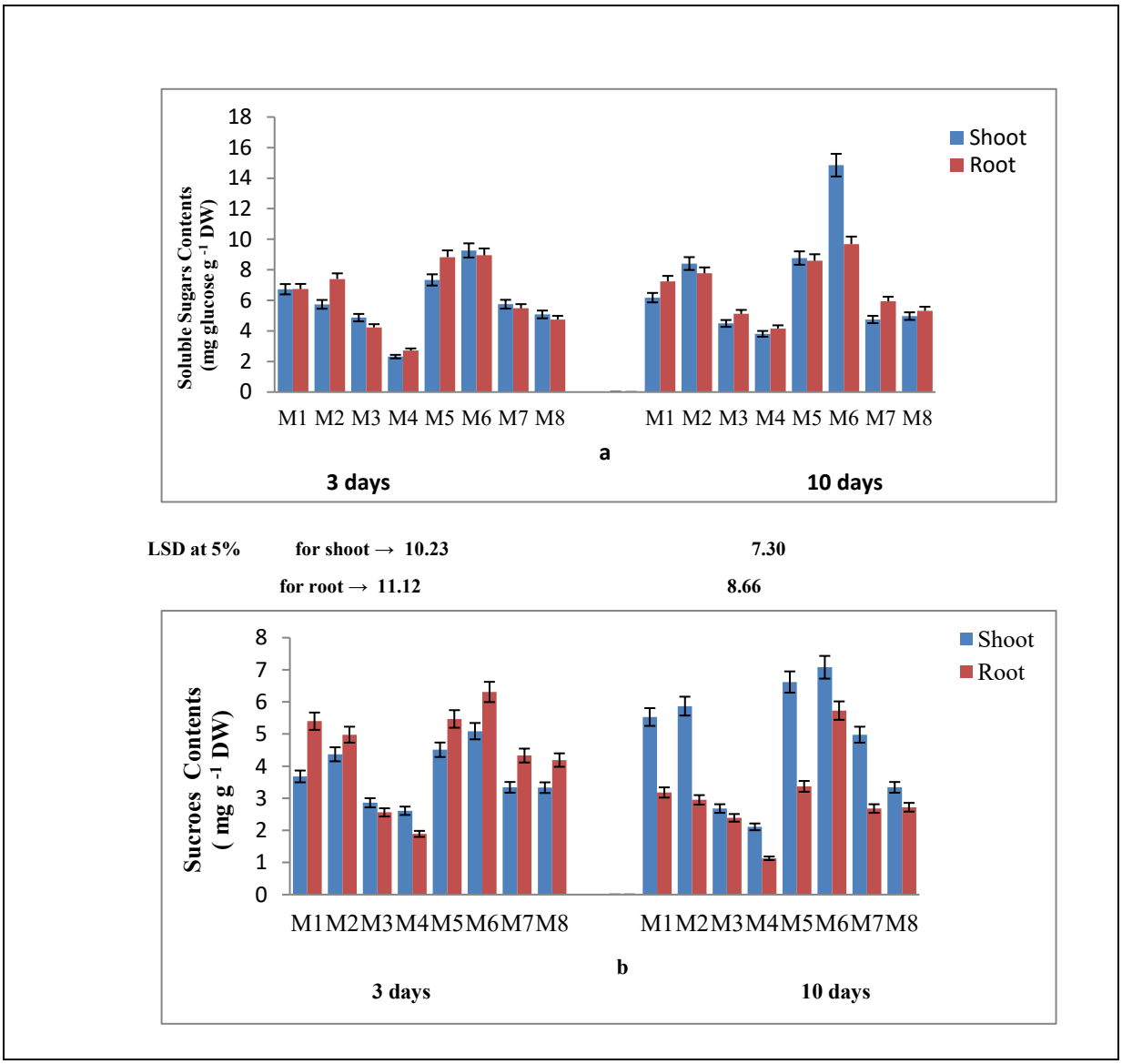
Changes in soluble sugars, sucrose and starch contents:

The changes of soluble sugars, sucrose and starch contents of *Lens culinaris* Medik. shoots and roots of regenerated plantlets are shown in Fig. 1 (a - c). High significant correlation had been showed between the photosynthetic pigments content and carbohydrate fractions (total soluble sugars, sucrose, and starch). However,

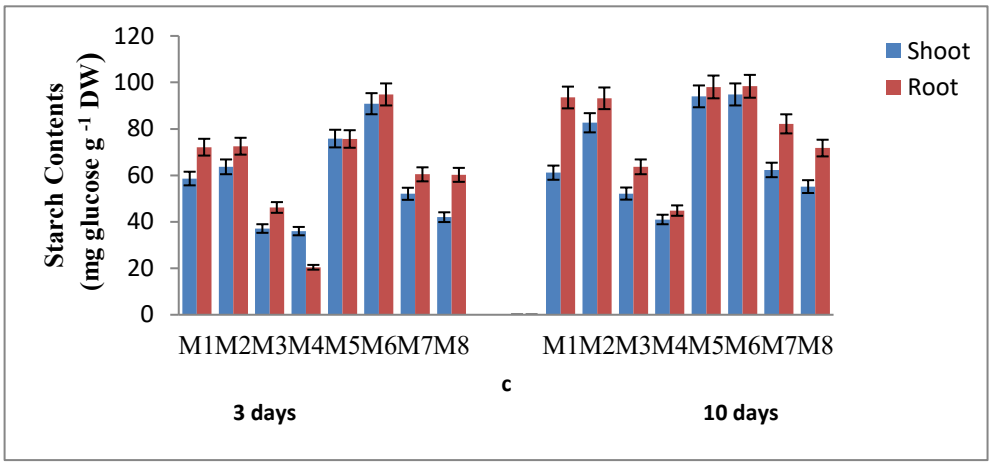
cultured medium amendment with 50 mM NaCl enhanced the synthesis of soluble sugars, which lead to increase in sucrose and starch content. On the other hand, increasing NaCl in the culture media up to 100 and 150 mM significantly reduced the tested carbohydrate fractions referred to control non-stressed.

The exogenous application of chitosan to *Lens culinaris* Medik. plantlets cultured media significantly enhanced the biosynthesis production of soluble sugars, sucrose and starch in roots and shoots compared to non stressed plantlets. The present data

illustrated in Figure 1 (a -c) and showed that the exogenous application of 0.1 mg L^{-1} chitosan potentially reduced the injury effect of salt stress. However when applying the hormone along with different salt levels, soluble sugars, sucrose and starch recorded further increment in contents of the plantlets grown on medium supplemented with 50 mM NaCl + chitosan (M_6). In addition, chitosan could ameliorate the 100 and 150 mM NaCl negative impact in the plantlet two parts during the experiment time compared to similar salted one.



LSD at 5% for shoot → 0.22 0.11
for root → 0.25 0.20



LSD at 5% for shoot → 7.23 6.25
for root → 7.18 6.86

Fig. 1: Effects of exogenous amendment of chitosan to MS culture media on soluble sugars (a), sucrose (b) and starch (c) contents of shoots and roots of *Lens culinaris* Medik. regenerated plantlets under normal and conditions of different salinity levels at 3 and 10 day harvesting time after application.

Where:

M_1= MS free CHT and NaCl (control)
M_2 = MS + 50 mM NaCl
M_3= MS + 100 mM NaCl
M_4= MS + 150 mM NaCl
M_5 = MS + 0.1 mg L^{-1}CHT
M_6 = MS + 50 mM NaCl + 0.1 mg L^{-1} CHT
M_7 = MS + 100 mM NaCl + 0.1 mg L^{-1} CHT
M_8 = MS + 150 mM NaCl + 0.1 mg L^{-1} CHT

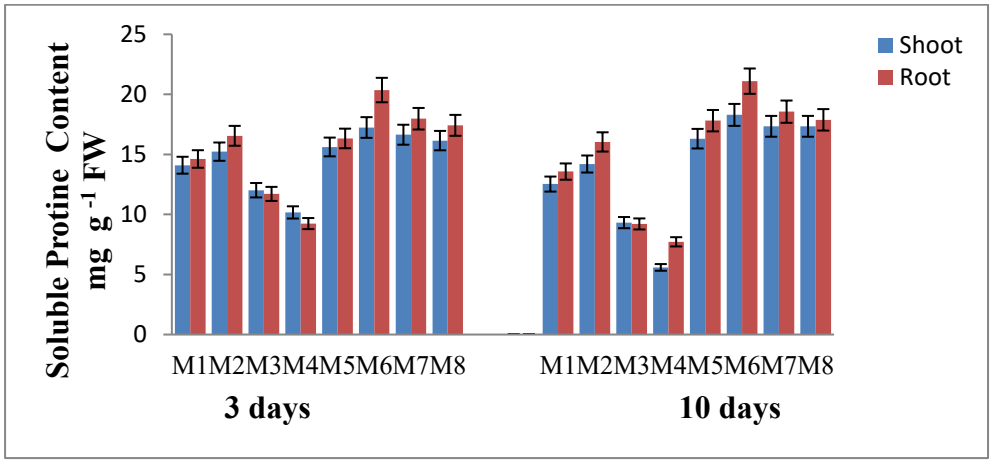
Changes in soluble protein

The effect of various concentrations of NaCl in the absence and presence of chitosan on the total soluble protein of *Lens culinaris* Medik. plantlets leaves and roots is represented in Fig. 2. The results indicated that the high concentrations of NaCl (100 and 150 mM) caused high significant decrease in the total soluble proteins content of plantlets shoots and roots below those of untreated ones.

On the other hand, the low concentration of NaCl (50 mM) caused opposite pattern of change in shoot and root of *Lens culinaris* Medik. plantlets. Application of chitosan alone and concomitant with all salinity levels caused significant increase in total soluble protein content in plantlets root and shoot of *Lens culinaris* Medik. compared to stressed or non stressed control. In general the inhibition of total protein contents by salt stress was partially alleviated by chitosan application.

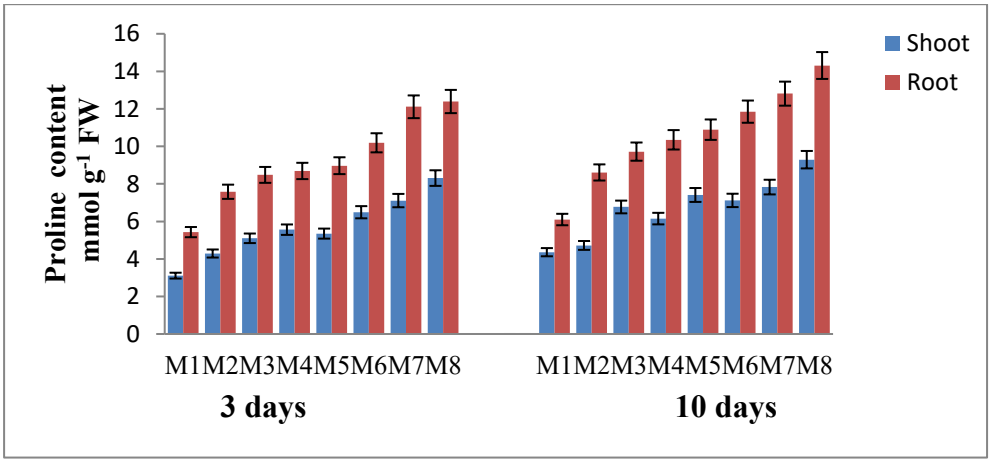
Changes in proline :

Concerning changes in the proline content after applying different NaCl levels alone or along with chitosan in shoot and root of *Lens culinaris* Medik. plantlets was represented in Fig. 3. Linear significant increment in proline content was recorded along with salt dose increments. As a marked increase in proline content was noted for plantlets cultured on modified MS with chitosan only compared with control, further enhancement in its content was scored in *Lens culinaris* Medik. plantlets cultivated on MS fortified with a combination of the chitosan combined with different salt levels (Fig. 3). The highest level of proline was estimated in M_8 plantlets compared to control non-stressed ones.



LSD at 5%	**for shoot →**	3.10	4.56
	for root →	3.62	4.94

Fig. 3: Effects of exogenous amendment of chitosan to MS culture media on soluble protine contents of shoots and roots of *Lens culinaris* Medik. regenerated plantlets under normal and conditions of different salinity levels at 3 and 10 day harvesting time after application.



LSD at 5%	**for shoot →**	1.14	1.01
	for root →	1.22	2.25

Fig. 3: Effects of exogenous amendment of chitosan to MS culture media on proline contents of shoots and roots of *Lens culinaris* Medik. regenerated plantlets under normal and conditions of different salinity levels at 3 and 10 day harvesting time after application.

Where:

M_1= MS free CHT and NaCl (control)
M_2 = MS + 50 mM NaCl
M_3= MS + 100 mM NaCl
M_4= MS + 150 mM NaCl
M_5 = MS + 0.1 mg L^{-1}CHT
M_6 = MS + 50 mM NaCl + 0.1 mg L^{-1} CHT
M_7 = MS + 100 mM NaCl + 0.1 mg L^{-1} CHT
M_8 = MS + 150 mM NaCl + 0.1 mg L^{-1} CHT

Changes of hydrogen peroxide and lipid peroxidation product content:

The effect of various concentrations of sodium chloride in absence and presence of chitosan on hydrogen peroxide (H_2O_2) and lipid peroxidation (malondialdehyde)

content of *Lens culinaris* Medik. regenerated plantlets are represented in Fig. 4 (a and b). Represented results revealed that H_2O_2 and malondialdehyde contents in shoot and root of plantlets were significantly increased at the three (50, 100 and 150mM) levels of salinity compared to control non-stressed. Treatment with chitosan alone and along with all salinity levels cause obvious reduction in H_2O_2 and TBARS contents in regenerated plantlets compared to non-stressed ones.

Salinity stress involves changes in various physiological and metabolic processes, depending on severity and duration of the stress, plant genotypes, environmental factors and ultimately inhibits crop production [29]. Initially salinity is known to represses plant growth manifested as osmotic stress, which is then followed by ion toxicity [29]. In most of the cases, the negative effects of salinity have been attributed to increase in Na and Cl ions in different plants hence these ions produce the critical conditions for plant survival by intercepting different plant mechanisms. Although both Na^+ and Cl^- are the major ions which produce many physiological disorders in plants, Cl^- is the most dangerous [30].

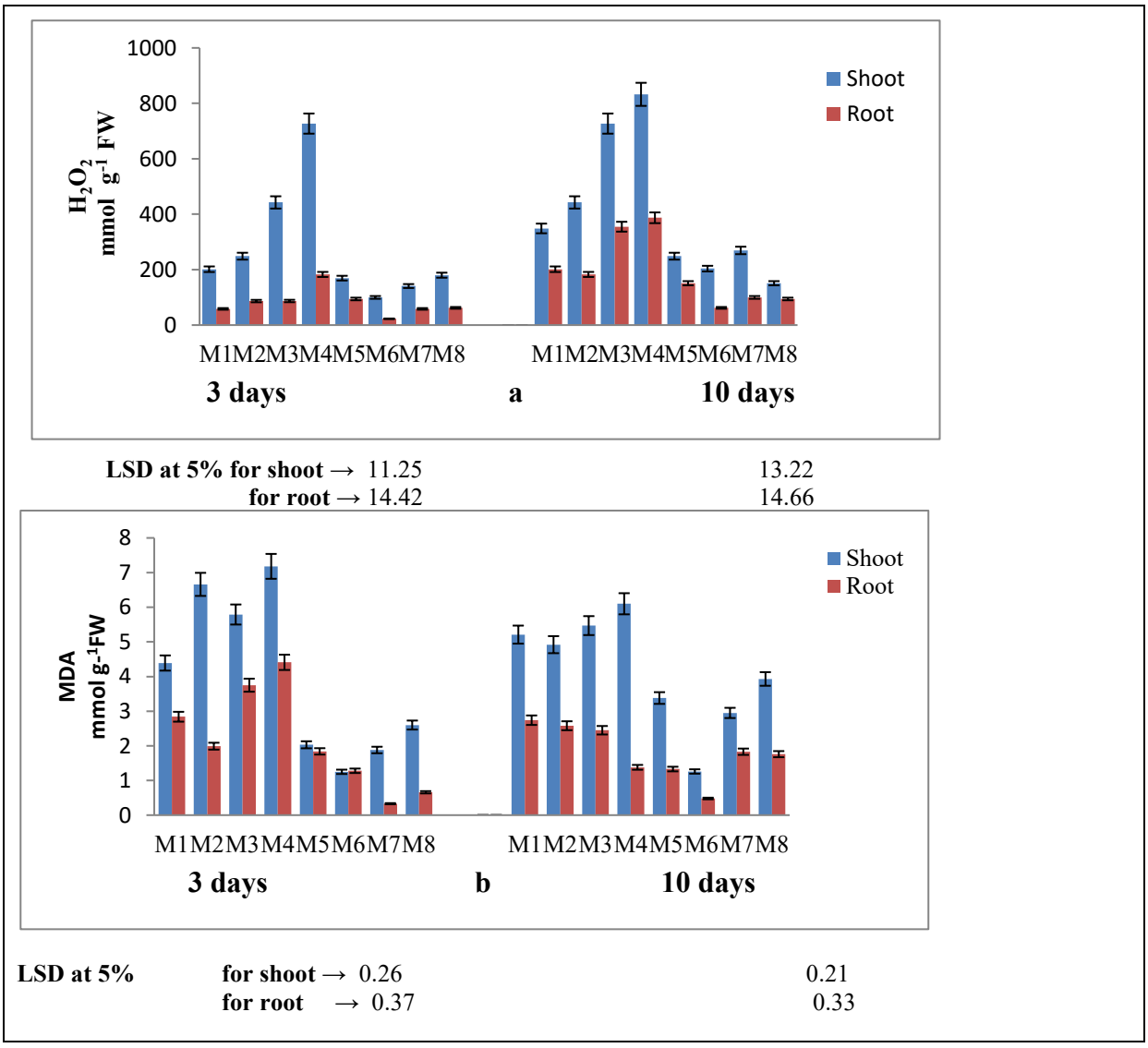


Fig. 4: Effects of exogenous amendment of chitosan to MS culture media on (a) hydrogen peroxide (H_2O_2) and (b) malondialdehyde (MDA) of shoots and roots of *Lens culinaris* Medik. regenerated plantlets under normal and conditions of different salinity levels at 3 and 10 day harvesting time after application.

Where:

M_1= MS free CHT and NaCl (control)	M_5 = MS + 0.1 mg L^{-1}CHT
M_2 = MS + 50 mM NaCl	M_6 = MS + 50 mM NaCl + 0.1 mg L^{-1} CHT
M_3= MS + 100 mM NaCl	M_7 = MS + 100 mM NaCl + 0.1 mg L^{-1} CHT
M_4= MS + 150 mM NaCl	M_8 = MS + 150 mM NaCl + 0.1 mg L^{-1} CHT

During the initial phases of salinity stress, water absorption capacity of root systems decreases and water loss from leaves is accelerated due to osmotic stress of high salt accumulation in plants, and therefore salinity stress is also considered as hyperosmotic stress [31]. The outcome of these effects may cause membrane damage, nutrient imbalance, altered levels of growth regulators, enzymatic inhibition and metabolic dysfunction, including photosynthesis, protein and nucleic acid synthesis which ultimately leads to plant demise [31 and 32].

Biochemical and molecular studies of salt stress responses in plants have revealed significant increases of reactive oxygen species (ROS), including singlet oxygen ($^1O_2$), superoxide (O^{2-}), and hydroxyl radical (OH •) and hydrogen peroxide (H_2O_2) [33]. Salinity tolerance may be defined as the ability of a plant to grow and complete its life cycle under stressful salt conditions like NaCl or with association of other salts [34].

Mechanisms of salt tolerance, not yet completely clear, can be explained to some extent by stress adaptation effectors that mediate ion homeostasis, osmolyte biosynthesis, toxic radical scavenging, water transport, and long distance response co-ordination [35]. However, attempts to improve yield under stress conditions by plant improvement have been largely unsuccessful, primarily due to the mutagenic origin of the adaptive responses. Therefore, a well-focused approach combining the molecular, physiological, biochemical and metabolic aspects of salt tolerance is essential to develop salt-tolerant crop varieties. Exploring suitable ameliorants or stress alleviant is one of the tasks of plant biologists.

Salinity stress is one of the most deleterious a biotic stress factors that affect the growth, productivity and physiology of plants. Salinity imposes negative effect on the plant growth by decreasing leaf water potential, inducing morphological and physiological changes, production of reactive oxygen species (ROS), increased osmotic stress, ion toxicity and by altering the biochemical processes. Salinity perturbs through its osmotic effect multitude of physiological processes including photosynthesis a process which is the main determinant of the dry matter accumulation and productivity of the crops.

Chlorophyll *a,* chlorophyll *b* and carotenoids are main photosynthetic pigments and play important role in photosynthesis. The changes in the amount of pigments were evaluated as the changes in photosynthesis. Negative correlation was recorded between pigments contents and salt levels, so they can be used as parameter for selection of tolerant and sensitive cultivars in crop plants [36]. On the other hand, changes in pigments contents were found to be affected by exposure time and salt concentration as well as plant species or genotypes [37].

Increased tolerance to salinity stress in crop plants is necessary in order to increase productivity with limited water supplies and high salinity. Tolerant genotypes respond to salinity stress with complex changes in their physiological and molecular status [37].

Carotenoids are necessary for photo protection of photosynthesis and they play an important role as a precursor in signaling during the plant development under a biotic/biotic stress. Decrease in carotenoids lead to degradation of $_{B}$-carotene and formation of Zeaxanthins, which are apparently involved in protection against photo-inhibition [38]. Nowadays, enhanced carotenoids contents in plants are of considerable attention for breeding as well as genetic engineering in different plants [39].

Agami [40] recorded an obvious decrease in maize photosynthetic pigments caused by NaCl stress when compared to the control. This result already observed by several authors on various crops, common bean [41], pea [42], sunflower [43] and lentil [44].

In addition, Rahdari [45] found that stressed lentil with lead acetate resulted in chlorophyll reductions and they explained that such loss in chlorophyll content can consequently lead to disruption of photosynthetic machinery. In the same line, stress generated by NaCl was founded to decrease the levels of chlorophyll fractions (chlorophyll *a*, *b* and total) and that of carotenoids in *P. vulgaris cv.* bronco plants [46]. In another study, Murakeozy *et al.,* [47] found significantly higher chlorophyll and carotenoids contents at 60 mM NaCl, and they suggested that relative water and carotenoids contents could be used as reliable selection criteria for salt tolerance in hot pepper.

Salinity disrupts many of plants cells physiological and biochemical processes in this regard; the decline in photosynthesis observed under salt stress can be attributed to stomata closure leading to a reduction of intercellular CO_2 concentration, or to non-stomata factors [48]. Non stomata restriction of net assimilation may originate from a reduced efficiency or regeneration capacity of ribulose-1,5-biphosphate (RuBP) carboxylase, a sensitivity of photosystem II (PSII) to NaCl, or a reduced leaf chlorophyll concentration [46].

Reduction in photosynthetic pigments amounts with NaCl application was suggested to be due to increasing in destructive enzymes called chlorophyllase or may be due to weakening of protein-pigment-lipid complex [49] . Previously, Assche and Clijsters [50]. explained decline in chlorophyll content under abiotic stress as due to inhibition of important enzymes, such as δ-aminolevulinic acid dehydratase and protochlorophyllide reductase associated with chlorophyll biosynthesis.

Soluble carbohydrates accumulation in plants has been widely reported as a response to salinity or drought, despite a significant decrease in net CO_2 assimilation rate [51]. Soluble sugar accumulation may be due to further transformation of starch to sugars or less consumption of carbohydrates by the tissues in saline conditions [52]. Carbohydrates accumulations under salt stress play a leading role in osmoprotection, osmotic adjustment, carbon storage, and radical scavenging [53]. Carbohydrates are supplied mainly through the process of photosynthesis and photosynthesis rates is usually lower in plants exposed to salinity and especially to NaCl [52].

In lentil, Sidari *et al.,* [54] indicated a lower content of total soluble sugars in presence of the highest salt concentration (250 mM) in castelluccio and eston cultivars compared to ustica and pantelleria cultivars, suggesting that salt tolerance ability of these two last landraces appears to be associated to the accumulation of osmolytes which improved their water status. Younis *et al.,* [55] found that increasing salt stress up to 120 mM NaCl in *Phaseolus vulgaris* seedlings growth medium resulted in Na^+ and CI^- ions accumulation in shoots and so inhibited the rate of photosynthesis as it is evident from

decrease in carbohydrate contents. Similar observations have been found in in *vicia faba* [56].

More recently, Abu-Muriefah [57] suggested that reduction in total carbohydrate in common bean plants induced by water stress treatments may be due to its inhibitory effect on photosynthetic activities, photosynthetic pigment concentrations. There is evidence that at low salt concentration salinity sometimes stimulate soluble sugar [58].

In this regard, Sadeghi and Shekafandeh [59] stated that until certain level of salt stress (EC: 6 dS m^{-1}) with increasing salinity, total soluble carbohydrates increased and then decreased in loquat plants. In the same line, Sharaf [60] reported that 50 mM NaCl was found to accommodate soluble carbohydrate and then significant decrease was recorded by applying higher levels (150 mM) in both shoots and root of different Egyptian lentil cultivars (giza4, 51 and 370).

In accordance with previous results, Kerepesi and Galiba [61] suggested that an accumulation of carbohydrates in plant may enhance the ability of plant to salt tolerance and might be a useful trait to select drought and/or salt-tolerant genotype. Parida and Das [53] suggested that carbohydrates such as sugars (glucose, fructose and sucrose) and starch accumulation under low salt stress levels was to accommodate the ionic balance in the vacuoles.

Among plants mechanisms to adapt salt stress osmotic adjustment plays a vital role in the resistance or tolerance of the plant to the constraint. Nedjimi [62] reported that contribution of total soluble sugars accumulation in *lygeum spartum* (Poaceae) to osmotic adjustment was significant, since the total soluble sugars content increased with an increase in salinity. Osmotic adjustment, which is necessary for growth in a saline environment, may be accomplished by accumulation of inorganic and organic solutes. Inorganic ions are believed to be sequestered in the vacuoles, while organic solutes are assumed to be compartmentalized in the cytoplasm to balance the low osmotic potential in the vacuole [63].

During salinity induced oxidative stress, several cytotoxic reactive oxygen species (ROS) are continuously generated in the mitochondria, peroxisomes and cytoplasm, which can destroy the normal metabolism through oxidative damage of proteins and nucleic acids [64] impeding the growth and development of a vast majority of crops.

A relationship between protein metabolism and NaCl stress in plants were well documented, salinity affects the metabolism of nitrogen containing compounds, protein synthesis and free amino acid pool composition [65]. Salinity decreases protein synthesis and increases its hydrolysis in many crop plants for instance, in lentil [54], common been [55], pigeon pea [66], and cucumber [67].

The decrease in protein content may be caused by enhanced protein degradation process because of increased protease activity [68] that is found to increase under stress conditions. Another explanation for protein degradation under saline environment has been reported due to decrease in the availability of amino acids and denaturation of enzymes involved in protein synthesis [69].

Under salt-stress, plants restrict the uptake of salt and adjust their osmotic pressure by the synthesis of compatible organic solutes. Compatible solutes are low molecular weight, highly soluble compounds that are usually nontoxic at high cellular

concentrations. These solutes include proline, sucrose, trehalose, and quaternary ammonium compounds such as glycine betaine, alanine betaine [70].

Proline, is one of most studied compatible solute playing a predominant role in protecting plants from osmotic stress [71 and 72]. Under salt stress, several functions are proposed for the accumulation of proline in tissues which include osmotic adjustment, carbon and nitrogen reserve for growth after stress resistance, detoxification of excess ammonia, stabilization of membranes, protecting photosynthetic activity and mitochondrial functions, and scavenging of free radicals [73]. However, the significance of proline accumulation in osmotic adjustment is still controversial and varies according to the species [74].

Many authors reported proline accumulation in the whole plants in field trials when exposed to salt stress [75]. In this regard, Cha-um and Kirdmanee [75] reported that proline was generally accumulated in osmotically-stressed sugarcane plantlets and played a key role in osmoregulation and antioxidant defense mechanisms. In addition, Panteleitchouk *et al.,* [76] studied and evaluated the effects of NaCl on carob plants grown on medium containing 0.05 M and 0.15 M NaCl during thirty days and compared them to plants grown in medium without NaCl. It was reported that the proline content was always higher in the NaCl-treated young plants compared to the control group. Similarly, proline accumulation under salt stress in different plants such as lentil [73], tomato [45] and jojoba plant [77] were recorded.

Proline accumulation can serve as a selection criterion for the tolerance of most species to stressed conditions [38]. In this regard, Chutipaijit *et al.,* [78] reviewed that proline accumulation normally occurs in the cytosol and suggested that it correlated with stress tolerance. Proline concentration has been shown to be generally higher in stress-tolerant plants than in stress-sensitive.

One of the biochemical changes occurring when plants are subjected to salt stress is the accumulation of reactive oxygen species (ROS) [79]. Reactive oxygen species can seriously disrupt normal metabolism through oxidative damage to lipids, protein and nucleic acids and damage membrane function [80].

Lipids play an important role as the structural constituent of most of the cellular membranes [53], which play a fundamental role in cell permeability [81]. Evidence suggests that membranes are the primary sites of salinity injury to cells and organelles [82] because ROS can react with unsaturated fatty acids to cause peroxidation of essential membrane lipids in plasma lemma or intracellular organelles. Peroxidation of plasma lemma components leads to the leakage of cellular contents, rapid desiccation, and cell death. Intracellular membrane damage can affect respiratory activity in mitochondria, causing pigment to break down and leading to the loss of the carbon fixing ability in chloroplasts [83]. It is well known that free radical-induced peroxidation of lipid membrane is a sign of stress induced damage at cellular level. Therefore, the level of malondialdehyde (MDA), produced during peroxidation of membrane lipids, is often used as an indicator of oxidative damage [84]. Lipid peroxidation was synchronized with increased salinity levels which had a relation with plants such as wheat [85], tomato [86] and mustard [89].

In recent decades, exogenous protectant such as osmoprotectants as proline, plant hormone as brassinosterioids, bio-stimulants as chitosan, antioxidants as ascorbic acid, signaling molecules as nitric oxide, polyamines as putrescine and trace elements as selenium have been found effective in mitigating the salt induced damage in plant [90

and 91]. These protectants showed the capacity to enhance the plant growth, yield as well as stress tolerance under salinity. Many compounds are being used to cope with the toxic effects of salinity among them chitosan treatments successfully overcame the toxicity generated by NaCl-stress and almost leveled the values with the control [92].

Mechanism of chitosan in plants has not been fully understood yet. However, there are many reports suggesting chitosan elicited a number of defense responses in the plants [93 and 94]. Chitin-specific receptors are present in plant cell membranes which are known to elicit defense responses. When treated with chitin-based treatment, plants activate their defense mechanism since they mimic compounds related to chitin-containing organism [95].

Chitosan have been used in the plants to confer resistance against abiotic stresses such as water deficit [10], salinity [11], and heavy metal toxicity [96]. Chitosan treatment enhances the closure of stomata through ABA synthesis and photosynthesis, enhances antioxidant enzymes by nitric oxide and hydrogen peroxide signaling pathways and induces the production of sugars organic acids, amino acids and other metabolites needed for osmotic stress-related adaptation, signaling of stress, and metabolism of energy [97].

Their capacity to scavenge ROS system and ultimately improved performance under stress has attracted researchers to offer a more varied application and continue to explore this novel biopolymer. In drought or dehydration stress, chitosan treatment alleviates the adverse effect caused by water stress by enhanced production of antioxidant enzymes, strengthening capability of water absorption through increased root growth and enhanced photosynthetic activities [98].

conclusion

Salt stress causes alterations in plant metabolism, including a reduced water potential, ion imbalances because it disturbs the uptake and translocation of mineral nutrients, oxidative stress and hormonal imbalance toxicity and sometimes severe salt stress may even threaten survival . In addition, salt stress can trigger various interacting events, including inhibition of enzymatic activities in metabolic pathways, and decreased carbon-use efficiency and decomposition of protein and membrane structures. Plantlets resulted from tissue culture techniques always were very delicate and must be carefully handle for laps of time to ensure successful regeneration protocol. Here the study took the challenge that subculture the resulted plantlets aged 3 weeks into MS only or MS invested with chitosan alone and /or with either of the three NaCl levels (at 50, 100 or 150 mM) can cope such harsh environments giving future hope to tolerate such sensitive cultivar against one of the most important challenge environmental clue. The present investigation reveals that 3-weeks old *in vitro Lens culinaris* Medik. plantlets sub cultured on MS and challenged with NaCl in comparison with those sub cultured on free MS, suffer from salt stress illness manifested as retardation in many of metabolic activities, concomitant with retardation in oxidative stress scavenging systems activities, imbalance in endogenous hormones and ion accumulation. This is corroborated with previous reports describe *Lens culinaris* Medik. plants as relatively sensitive to salt stress . However, this reduction in most *in vitro* plantlets parameters was alleviated in MS medium invested with (0.1 mg L^{-1} chitosan alone which was found to improve the cultured plantlets performance as manifested from the marvelous increment in estimated same previous parameters. Therefore, the study hypothesis was that chitosan could alleviate salt stress negative impact of *in vitro Lens culinaris* Medik.

regenerated plantlets *via* different collaborated strategies and enhance plantlets tolerance. The collected promise data points to that chitosan successfully tolerate *in vitro Lens culinaris* Medik. regenerated plantlets to face harsh salt condition.

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